Nuclear Magnetic Resonance Studies of Frozen Aqueous Solutions[1]

J. E. Ramirez,[2] J. R. Cavanaugh,* and J. M. Purcell

Eastern Regional Research Center,[3] Philadelphia, Pennsylvania 19118

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The relatively narrow ^1H nmr absorptions which arise apparently from mobile water molecules within frozen aqueous solutions were studied for frozen solutions of selected acids, bases, salts, amino acids, polypeptides, and proteins. The changes in integrated intensity of the resonances with temperature can be interpreted in terms of the known phase diagrams of some of the systems studied. Observations concerning amino acids, polypeptides, and a protein suggest that the interpretation of the narrow nmr absorptions as representing water of hydration may be inaccurate. In particular, studies of the ^1H and ^{19}F resonances of the frozen KF–protein–H_2O system indicate that the narrow resonances arise from a solute-rich water phase that could best be described as a liquid-like aqueous phase.

A series of investigations[4] has been reported on the surprisingly narrow proton resonance observed from frozen aqueous solutions of a variety of macromolecules. The resonances observed could not be assigned either to the protons in the ice lattice or to those belonging to the macromolecule but the intensity was proportional to the amount of protein present. It was suggested that these resonances arose from water molecules associated in such a way with the macromolecules as to be prevented from joining the ice lattice; that is, they were interpreted as arising from "water of hydration." Our own interests in the interaction of amino acids in aqueous solutions[5] prompted us to investigate this phenomenon further. We report here the results of that investigation.

Experimental Section

The nmr spectra were recorded on a Varian Associates DA-60-IL spectrometer[6] equipped with a variable temperature probe and operated at 60 and 56.4 MHz. Relative integrated intensities were calculated at least in duplicate from four or more recordings taken with alternating upfield and downfield sweep. The values were adjusted according to the attenuation levels as set on the integrator unit. The temperature in the probe was measured directly by means of a thermistor thermometer placed inside an empty nmr sample tube at the location of the transmitter–receiver coils. The probe assembly was fitted with a standard pressure cap through which the leads from the thermistor were led to a resistance bridge for measurement. The temperature could be measured to within 0.5° and remained stable to ±1° during the course of the nmr measurements. All compounds studied were of the highest quality obtainable commercially and were used without further purification.

Results and Discussion

Two Component Systems. In order to provide a background against which to interpret the narrow resonances

TABLE I: Comparison of the Freezing Points of Aqueous Solutions Determined by Nmr with Those Taken from the Phase Diagrams

Solute[a]	Freezing point determined by nmr[b]	Freezing point of lowest-solute-concentration eutectic[c]
NaCl	−18	−21
KCl	−8	−11
$Na_2S_2O_3$	−21	−22
NaOH	−30	−28
HCl	−85	−85
CH_3COOH	−26	−26
$CH_2ClCOOH$	−19	−14
$CHCl_2COOH$	−71	−70
CCl_3COOH	−70	−50

[a] Initial solute concentration was 5% by weight. [b] In °C. The freezing point was taken to be the temperature at which the narrow water resonance disappeared and could be estimated to within 2°. [c] In °C. Values taken from ref 8. Values for the substituted acetic acids were from ref 9.

of frozen solutions of macromolecules, a survey was undertaken of frozen solutions of some low molecular weight compounds. A variety of samples, listed in Table I, were found to yield narrow proton resonances[7] at −30° when prepared in accordance with the published procedure.[4] For example, frozen solutions of HCl showed very narrow resonances even at temperatures down to −85°. There were two important differences, however, between the behavior of these two component systems and those containing proteins. First, the integrated intensity of the narrow resonance decreased with decreasing temperature. Second, as the temperature was lowered, the narrow resonance eventually disappeared at a temperature which was characteristic of the solute under investigation and independent of the initial solute concentration.

The phenomenon was generally reversible; heating the sample resulted in the reappearance of the narrow signal of the same intensity. Moreover, the results were identical whether the sample was cooled in the probe from room temperature or heated in the probe after having been quenched in liquid nitrogen.

The behavior of these systems is readily explicable in terms of the phase diagrams of the binary mixture. As the temperature is lowered to a point beyond that of the initial freezing point of the mixture, ice crystals form throughout the sample. This results in an increase in the concentration of the solute in the liquid remaining until equilibrium is reestablished at the new lower temperature. The diminished nmr signal represents the water remaining in the liquid phase. As the temperature is successively lowered, this process repeats until the liquid reaches the composition of the eutectic. At this point, the entire mixture freezes and the narrow resonance disappears.

In Table I, we compare for a variety of binary systems the final freezing points as determined by the disappearance of the narrow nmr signal with the temperature of the eutectic of lowest-solute concentration as taken from the phase diagrams. The agreement is excellent.[10]

It may be noted in passing that the nmr techniques afford a quick and convenient method for constructing the phase diagram up to the composition of the first eutectic. If it is assumed that the signal response is independent of temperature, the relative area at any given temperature yields the appropriate compositional data for the liquid phase at that temperature. We have confirmed this result with measurements on aqueous solutions of HCl and the acetic acids.

TABLE II: Temperature of Disappearance of Narrow Resonance of Frozen Aqueous Solutions

Solute	Initial concn[a]	Temp[b]
Glycine	10	~0
L-Alanine	10	~0
L-Valine	5	~0
L-Methionine	5	~0
L-Serine	5	−35
L-Threonine	5	−40
L-Lysine HCl	7	−60
Poly-L-lysine HCl	7	−60
L-Tyrosine, sodium salt	3	−50
Poly-L-tyrosine, sodium salt	3	−50

[a] In wt %. [b] In °C. The resonances from these samples were sufficiently broad (500 to 1500 Hz width at half height) that the temperature of disappearance could not be determined to better than 5°.

Amino Acids and Polypeptides. Amino acids in aqueous solutions containing approximately equimolar amounts of either acid or base display narrow resonances at low temperature. A variety of experiments were conducted at −40° and without exception all amino acids studied yielded this result. Perhaps this is not so surprising in view of the fact that NaOH and HCl solutions themselves exhibit this behavior.

A study of amino acids dissolved in water was next attempted. Consideration was limited to those amino acids which were relatively soluble at room temperature. The results are summarized in Table II. Included are data on two polypeptides together with their respective amino acid solutions.

Of the six amino acids soluble enough for this study, only two yield aqueous solutions that exhibit the narrow resonances at low temperature. Significantly, both these molecules contain an -OH group in addition to the amino acid functional groups. Perhaps the hydrophilic -OH group plays a similar role to that shown by the H^+ and OH^- ions in frozen acid and base solutions of amino acids.

The results on the polypeptides are noteworthy in two respects: (1) the intensity of the narrow signal decreases with decreasing temperature precisely like the other systems described; and (2) the temperature of disappearance of the resonance is the same for the polypeptide as for the respective amino acid.[11] These latter results are shown in Table II. While undue significance should not be attached to the coincidence of the numbers obtained, these results suggest that whatever mechanisms are involved in the origin of the narrow resonance displayed by the frozen solutions of the amino acids, those same mechanisms are operative in the polypeptide systems. Additional mechanisms need not be invoked to interpret the polypeptide results. Even though these mechanisms are clearly not well understood, the results obtained on the simple systems point to an interpretation based on a solute-rich liquid phase present within the frozen solutions.

Proteins. Frozen solutions of proteins, as reported previously[4] and as confirmed by our own experiments, behave radically differently from the systems discussed thus far. In the first instance, while the line widths of the narrow resonances increase with decreasing temperature, the integrated intensity remains constant. Second, the signals persist at the lowest temperatures obtainable (−100° on our spectrometer). The attractive interpretation ad-

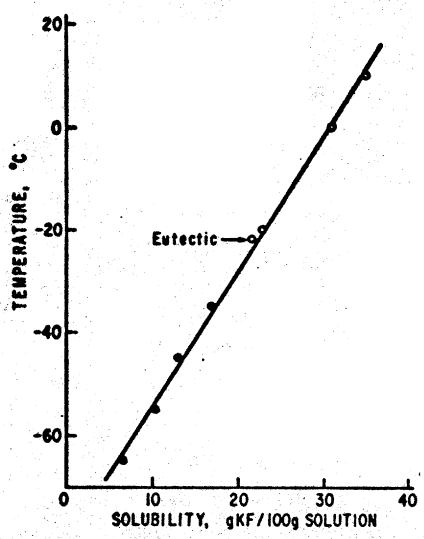

Figure 1. "Nonfrozen" KF associated with "nonfrozen" water *vs.* temperature, plotted in terms of solubilities (closed circles). Solubility of KF in H_2O *vs.* temperature, as taken from the phase diagram (open circles).

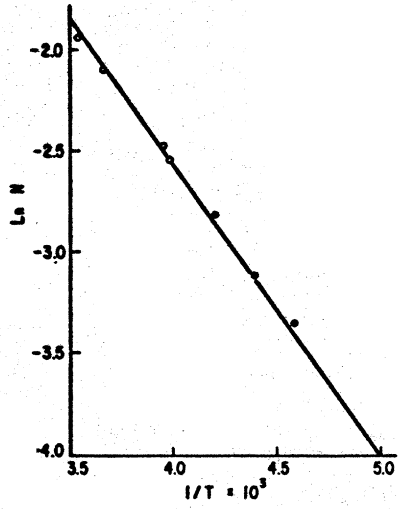

Figure 2. Natural logarithm of the solubilities of KF in H_2O expressed in mole fraction units *vs.* $1/T$. Closed circles, "solubility" data derived from the present study; open circles, actual solubilities.

vanced[4] was that the narrow resonance arose from water of hydration surrounding the macromolecule which was prevented from joining the normal ice lattice because of its interaction with the macromolecule. This interpretation had the added utility of providing information on hydration of macromolecules in a variety of situations. However, some of our own observations, especially our studies of the ternary system, H_2O–KF–bovine serum albumin (BSA), and some pertinent literature related to hydration of macromolecules, raise a number of questions regarding this interpretation.

In the first consideration, the protein systems studied are highy complex. The aqueous solutions contain in addition to the protein, added salts, counterions, and, possibly, excess acid or base. Any of these components themselves can give rise to narrow resonances in frozen aqueous solutions. Even ternary systems are poorly understood let alone the heterogeneous interface between the protein and the solvent water. It is very possible that the intermolecular interactions responsible for the binary phase diagrams operate analogously in these more complex systems and because of the diversity and heterogeneity of the protein–water interactions combine to produce the behavior noted.

Secondly, in a study of frozen solutions of BSA as a function of pH, the integrated intensity did not vary according to what would be expected of water of hydration. Thus, as reported previously[4] and confirmed by our own observations, the intensity of the narrow resonance decreased with decreased pH. However, lowering the pH brings about a partial unfolding of BSA and an increase in the hydration of the molecule by more than a factor of 3 as witnessed by small angle X-ray scattering results.[12] Consequently, an explanation of the decreased intensity of the narrow resonance with increased acidity must be searched for in a direction other than hydration.

Finally, an interpretation of the narrow resonance based on a solute-rich liquid phase is strongly indicated by our experiments on the KF–H_2O–BSA system. Both ^{19}F and ^{1}H nmr spectra were obtained on frozen solutions of KF–H_2O–BSA of varying composition. The ^{1}H spectrum was as described previously for BSA solutions,[4] the KF having no observable effect on the results. A narrow resonance

TABLE III: Amounts of "Nonfrozen" KF[a] in Frozen KF–H_2O–BSA Solutions

Temp[b]	KF relative to BSA[c]	KF relative to "nonfrozen" water[d]
−35	0.078 ± 0.007	0.204
−45	0.057 ± 0.009	0.150
−55	0.044 ± 0.005	0.117
−65	0.027 ± 0.001	0.071

[a] The amounts are calculated as the wt % of KF corresponding to the integrated intensity of the ^{19}F narrow resonance. [b] In °C. [c] Wt % of KF divided by the wt % BSA in the sample. Error limits are average deviations over several samples with varying KF–H_2O–BSA composition. [d] Wt % of KF divided by the wt % of "nonfrozen" water corresponding to the integrated intensity of the ^{1}H narrow resonance. Equivalently, column 2 divided by 0.38 g of "nonfrozen" water/g of BSA.

was also observed in the ^{19}F spectra of the frozen solutions with the following properties: (1) no narrow resonance was observed in the absence of BSA; (2) the intensity of the signal decreased with decreasing temperature, although the intensity of the narrow proton resonance remains constant; (3) at a constant BSA concentration, the intensity of the resonance was independent of the KF concentration above a required minimum indicated by the relative amounts as shown in Table III (see below); and (4) at a given temperature, the intensity of the ^{19}F narrow resonance was directly proportional to the BSA concentration.

The constant of proportionality obtained by dividing the integrated ^{19}F intensity expressed as weight % by the BSA concentration, measures the amount of "nonfrozen" KF associated with the BSA molecule at a given temperature. These results are presented in Table III as a function of temperature. Also in Table III, the results are expressed as the amounts of "nonfrozen" KF relative to the "nonfrozen" water associated with the BSA molecule.

The amount of "nonfrozen" water associated with BSA is constant over the low temperature range measured. On the other hand, the amount of "nonfrozen" KF, as seen from Table III, diminishes with decreasing temperatures. As the temperature is lowered, less and less "nonfrozen" KF is associated with a constant amount of "nonfrozen" water. The phenomenon is strikingly characteristic of a

solubility process; that is, as the temperature is lowered, less and less KF can be accommodated in the liquid-like water phase. In other words, as the KF–H_2O–BSA solutions are frozen, part of the water remains in a liquid-like phase and a portion of the KF present "dissolves" in the liquid water, the solubility decreasing with decreasing temperature.

These solubility data are plotted in Figure 1 in terms of grams of KF in 100 grams of KF–H_2O solution. The closed circles in the lower left-hand portion of the graph are the solubilities of the "nonfrozen" KF derived from the present experiments. The open circles in the top right-hand portion of the graph are *actual* solubilities of KF in water, taken from the phase diagram.[8] The point at 21.5 g of KF/100 g of solution corresponds to the eutectic of lowest-solute concentration, melting point of −21.8°. The straight line is the linear least-squares fit of all eight points.

The continuity of the present solutility data and the actual solubilities above the eutectic temperature is truly remarkable. Yet what is perhaps even more unexpected is that the combined data fit a simple thermodynamic relationship. Thus, for a substance whose heat of fusion, ΔH, is independent of temperature, the solubility, N, expressed in mole fraction units, is given by[13]

$$\ln N = \frac{\Delta H}{RT} + C \qquad (1)$$

where C is a constant of integration. For compounds obeying this relationship, $\ln N$ will be a linear function of $1/T$.

Figure 2 illustrates for the combined data how well this linear relationship is met.[14] Not only does the solubility property of the liquid-like water phase appear to extend continuously beyond the eutectic temperature but the solubility phenomenon itself is describable in proper thermodynamic terms.

Conclusion

The appearance of a relatively narrow ^1H nmr absorption in frozen protein solutions is as puzzling as it is noteworthy and raises important questions concerning the nature of the protein–water interactions that give rise to a "mobile" water phase at low temperature. Even frozen binary mixtures give rise to narrow resonances but these cases find a straightforward interpretation in terms of the binary system phase diagrams. However, even here the solute–solvent interactions that determine the composition and melting point of the eutectic mixtures are not well understood. It is perhaps not so surprising then that the extremely complex protein–water interactions are also poorly understood. Even though a detailed knowledge of this phenomenon is lacking, an explanation in terms of water of hydration appears inadequate. Rather, the results on the KF–H_2O–BSA system suggest an interpretation based on a liquid water phase, rich in protein concentration and capable of acting like a true thermodynamic liquid phase.

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References and Notes

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(2) Research performed under tenure of an NRC–ARS Postdoctoral Research Associateship.
(3) Agricultural Research Service, U. S. Department of Agriculture.
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(6) Mention of commercial products does not constitute an endorsement by the U. S. Department of Agriculture over others of a similar nature not mentioned.
(7) The widths of the narrow water resonances depended on the nature of the solute and the temperature. For these binary mixtures, they were typically on the order of a few hundred Hz but ranged down to less than 20 Hz for solutions of HCl at −35°.
(8) A. Seidell, "Solubilities of Inorganic and Metal Organic Compounds," 4th ed, W. F. Linke, Ed., Vol. I, II, American Chemical Society, Washington, D. C., 1958, 1965.
(9) S. U. Pickering, *J. Chem. Soc.*, **67**, 664 (1895).
(10) The discrepancy for CCl_3COOH may arise from the tendency of the CCl_3COOH solutions to supercool. It was difficult to assess the freezing point both from the nmr experiment and visually. Consequently, the value we report (and/or the literature value) may be substantially in error.
(11) Reference 4 also reports the diminishing intensity of frozen polypeptide solutions with decreasing temperature but does not report on the disappearance of the narrow resonance at some temperature.
(12) V. Luzzati, J. Witz, and A. Nicolaieff, *J. Mol. Biol.*, **3**, 379 (1961).
(13) See, for example, "Thermodynamics," G. N. Lewis and M. Randall, Ed., 2nd ed, McGraw-Hill, New York, N. Y., 1961, p 227 ff.
(14) The point that fits most poorly at the lowest temperature corresponds to the resonance of the lowest signal intensity and poorest signal-to-noise ratio.